REMARKS

Reconsideration of the application is requested in view of the Amendments to the claims and the remarks presented herein.

The claims in the application are claims 1 to 6, 14, 15, 22, 24 and 25, all other claims being cancelled. Cancellation of claim 23 obviates the rejection thereof and claim 14 has been amended as suggested by the Examiner to obviate the rejection thereof.

All the claims were rejected under 35 U.S.C. 112, first paragraph as being based on a non-enabling specification for treatment of the disorders recited therein, namely treating disorders of central or peripheral nervous systems. The Examiner states there is no correlation between monoamine oxidases and lipidic peroxidation and modulating activity vis-à-vis sodium channels for any disorders, much less those recited in the claims.

Applicants respectfully traverse this ground of rejection since it is believed that the specification is enabling for the claimed methods. On pages 171 to 173 there is test data showing inhibitory activity on the bond of a specific ligand of ABO-B[^{3}A] Ro 19-637 and tests on the cerebral sodium channels. Applicants are submitting herewith an abstract of a Stoll et al. article [Life Sci., 1994, 55(25-26): 2155-63] showing that inhibition of MAO-B is useful for the treatment of Alzheimer and Parkinson's diseases. Also enclosed is an article by Beal [Neuro scientist, Vol 3, No. l, 1997, p. 21 to 27]

showing that lipid peroxidation is useful for the treatment of Alzheimer's disease, Parkinson's disease, Huntington's disease, ALS and cerebral degeneration.

The following specific compounds are within the scope of claims 1 and 22.

Example 23: 2-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]-4-oxazoleethanol:

example 26: 2,6-ditert-butyl-4-(4-{2-[methyl(2-propynyl)amino]ethyl}-1,3-oxazol-2-yl)phenol:

example 27: [{2-[2-(3,5-ditert-butyl-4-hydroxyphenyl)-1,3-oxazol-4-yl]ethyl}(methyl)amino]acetonitrile:

example 28: 3-[{2[{2-{2-(3,5-ditert-butyl-4-hydroxyphenyl)-1,3-oxazol-4-yl]ethyl}(methyl)amino]propanenitrile:

example 29: 2,6-ditert-butyl-4-{4-[2-(1-piperazinyl)ethyl]-1,3-oxazol-2-yl}phenol hydrochloride:

example 319: 2,6-di*tert*-butyl-4-[4-(hydroxymethyl)-1,3-thiazol-2-yl]phenol

example 346: 2,6-ditert-butyl-4-{4-[(methylamino)methyl]-1,3-thiazol-2-yl}phenol hydrochloride.

The test data in the application clearly is enabling for the scope of the present claims.

- Compounds 26 to 29 have an inhibitory activity of the monoamine oxydases activity (MAO-B) as they show an IC_{50} lower than 10 μM (page 171), lines 27

to 28 of the English translation). Now it's known that MAO-B is involved in particular pathologies as disorders of the central or peripheral nervous systems such as for example neurological diseases where Parkinson's disease, cerebral or spinal cord traumatisms, cerebral infraction, sub arachnoid haemorrhage, epilepsy, ageing, senile dementia, Alzheimer's disease, Huntington's chorea, amyotrophic lateral sclerosis, peripheral neuropathies, pain can in particular be mentioned as noted in the above cited art.

- Compounds 23, 26 to 29 have an inhibitory activity of the lipidic peroxidation as they show an IC_{50} lower than 10 μM (pages 172, lines 19 to 21 of the English text). This inhibitory activity of compounds 23, 26 to 29 is determined by measuring their effects on the degree of lipidic peroxidation, determined by the concentration of malondialdehyde (MDA). The MDA produced by peroxidation of unsaturated fatty acids is a good indication of lipidic peroxidation (H Esterbauer and KH Cheeseman, *Meth. Enzymol.* (1990) **186**: 407-421). The inhibition of the lipidic peroxidation indicates that Applicants'compounds have anti-oxidative properties. The advantage of the anti-oxidative properties are well understood in Parkinson's disease: in this pathology, the degradation of loss of the dopaminergic neuron is due to an oxidizing stress due to the reactive oxygen sepcies (page 2, lines 9 to 12 of the English text);

- Compounds 26, 28 and 29 act as modulators of the sodium channels and show an IC_{50} lower than 3,5 μM (page 173, lines 25 to 27 of the English translation). The test consists in measuring the interaction of the compounds vis-à-vis the bond of tritiated batrachotoxin on the volatge dependent sodium channels according to the protocol described by Brown (*J. Neurosci.* (1986), **6**, 2064-2070). The character of the modulator of the sodium channels is very useful for therapeutic indications such as the treatment or prevention of pain.

Therefore, it is deemed that the claims are based on an enabling disclosure and withdrawal of this ground of rejection is requested.

With respect to the obviousness double-patenting rejection, Applicants are submitting herewith a terminal disclaimer with respect to application Serial No. 10/681,002 and PTO form 2038 for the disclaimer fee there obviating this ground of rejection.

In view of the amendments to the claims and the above remarks, it is believed that the claims clearly point out Applicants'patentable contribution. Therefore,

favorable reconsideration of the application is requested.

Restpectfully submitted,
Hedman and Costigan

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CAM:mlp
Enclosures

REVIEW

Oxidative Damage in Neurodegenerative Diseases

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Increasing evidence has implicated oxidative damage in the pathogenesis of neurodegenerative diseases. The major source of free radicals in the cell is the mitochondria. Peroxynitrite is formed by the reaction of superoxide with nitric oxide, and it produces both oxidative damage and protein nitration. Mutations in CuZn superoxide dismutase associated with familial ALS may result in increased $^{\cdot}OH$ radical generation or in increased reactivity with peroxynitrite to nitrate proteins. There is evidence for increased oxidative damage in Alzheimer's disease and Parkinson's disease in neurons undergoing neurodegenerative changes. A role for oxidative damage in Parkinson's disease toxicity and in Huntington's disease is supported by studies in animal models. Improved antioxidant therapies may prove useful in slowing or halting the progression of neurodegenerative diseases. NEUROSCIENTIST 3:21–27, 1997

KEY WORDS Free radicals, Peroxynitrite, Alzheimer's, Parkinson's, Huntington's, Amyotrophic lateral sclerosis

The term "neurodegenerative diseases" implies that there is no exact knowledge of the cause or pathogenesis of these diseases. The neurodegenerative diseases are exemplified by Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), and cerebellar degenerations. The neuropathological features of these illnesses tend to be symmetrical and involve degeneration of circumscribed groups of neurons that may be functionally or neuroanatomically connected. Typically, the pathological process is one of slow involution of cell bodies and processes that may be disrupted by cytoskeletal abnormalities, without any intense inflammatory reaction.

The etiology of these illnesses is under intense investigation. Major advances have been spurred by molecular biology, which has led to the identification of genes associated with familial forms of several neurodegenerative diseases. It is, however, unclear how the genetic defects contribute to neuronal degeneration. Much interest has focused on oxidative damage as a potential contributor to the pathogenesis of these illnesses.

Oxidative Damage

All aerobic organisms are continually exposed to oxidative stress. Oxidation and reduction reactions involve the transfer of electrons, which can lead to the generation of free radicals. A free radical is any species that contains one or more unpaired electrons (1). Examples of free radicals are superoxide ($O_2^{\cdot-}$), hydroxyl ($^{\cdot}OH$), and nitric oxide ($NO^{\cdot}$) (Fig. 1). Most free radicals are unstable reactive species that can extract an electron from neighboring molecules to complete their own orbital. This leads to oxidation of neighboring molecules. A variety of critical biological molecules, including DNA, cellular proteins, and membrane lipids, are subject to oxidative damage. The reaction of free radicals with lipids can initiate chain reactions leading to lipid peroxidation.

The key mediators of oxidative damage in vivo are debated. The most reactive oxygen species is $OH^{\cdot}$, which reacts at near diffusion-limited rates within a few angstroms of the site where it is produced. Much interest has focused on the hydroxyl radical as the prime mediator of cell damage. In contrast, superoxide can cross membranes and may be able to act at a distance; however, it is much less reactive than the hydroxyl radical. It is capable of inactivating some enzymes containing iron-sulfur clusters, including aconitase, 6-phosphogluconate dehydratase, branched chain amino acid dehydratases, and complex I of the electron transport chain (2).

Superoxide radical is normally converted by superoxide dismutase (SOD) to H_2O_2. This is an important physiological antioxidant mechanism. H_2O_2 crosses cell membranes and can inactivate a few cell enzymes. However, H_2O_2 reacts with transition metals to generate hydroxyl radicals. Iron or copper have loosely bound electrons that can accept and donate electrons and, thereby, promote redox reactions. Fenton-type reactions occur when Fe^{2+} donates an electron to H_2O_2 to form the highly reactive hydroxyl radical. One source of free iron

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$$O_2 \xrightarrow{+e^-} O_2^{\cdot} \xrightarrow[+2H^+]{+e^-} H_2O_2 \xrightarrow{+e^-} OH^{\cdot} + OH^- \xrightarrow[+2H^+]{+e^-} 2H_2O$$

$$O_2^{\cdot} + O_2^{\cdot} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

$$H_2O_2 + Fe^{2+} \longrightarrow OH^{\cdot} + OH^- + Fe^{3+}$$

$$O_2^{\cdot} + NO^{\cdot} \longrightarrow ONOO^- \longrightarrow OH^{\cdot} + NO_2^{\cdot}$$

Fig. 1. Examples of free radicals found under physiological conditions.

is the $O_2^{\cdot}$ reaction with iron-sulfur clusters, which leads to oxidation of Fe^{2+} to Fe^{3+}. The oxidized cluster then loses Fe^{2+} because Fe^{3+} is more tightly bound to the sulfide ligands than Fe^{2+} (2). The site specificity of oxidative damage to DNA and proteins is thought to result from site-specific iron-binding properties of these macromolecules. Oxidation reactions are, therefore, influenced by the regional concentrations of transition metals. The conversion of iron from its oxidized (Fe^{3+}) to its reduced (Fe^{2+}) state promotes the formation of a cascade of free radicals. Iron in the cell is usually bound to transferrin or ferritin, which maintain it in a nonreactive state.

Another compound of recent interest is peroxynitrite. The interaction of NO with superoxide radical leads to the formation of peroxynitrite ($ONOO^-$) (3). This reaction occurs at an extremely fast rate of $6.7 \times 10^9\ M^{-1}\ S^{-1}$, which is threefold faster than the rate of dismutation of superoxide by SOD. Therefore, it depends on the concentrations of superoxide and NO in the cell. Both superoxide and NO can be produced by nitric oxide synthase (NOS) (4). Peroxynitrite itself is a highly reactive oxidizing agent that can cause tissue damage by an active intermediate that acts like an $OH^{\cdot}$ radical. The formation of peroxynitrite does not require transition metals (5). At physiological pH, peroxynitrite may be able to diffuse over several cell diameters to produce cell damage by oxidizing lipids, proteins, and DNA. Recent work has shown that activated macrophages produce peroxynitrite (6). Peroxynitrite can react with CuZnSOD to form nitronium ion, which then nitrates tyrosine residues (7). Therefore, SOD may be protective by scavenging superoxide before it reacts with NO to form peroxynitrite.

Some mechanisms serve to limit free radical production and oxidative stress, including the localization of oxidative phosphorylation to mitochondria, where there are abundant free radical scavenging mechanisms. Also, several antioxidant enzymes, including SOD, produce H_2O_2, which is then eliminated by either catalase or glutathione peroxidase. Some cellular antioxidant molecules, such as alpha-tocopherol (vitamin E), ascorbate, and glutathione can react directly with free radicals and spare other critical intracellular molecules. Normally, there is an equilibrium between free radical formation and antioxidant defense mechanisms. An imbalance leads to oxidative stress.

The two major intracellular sources of free radicals are prostaglandin synthesis and mitochondria. The synthesis of prostaglandins and leukotrienes is accomplished by peroxidase and lipoxygenase enzymes. These enzymes can abstract electrons from NADH or NADPH. The resultant radical forms of the nucleotides can then be oxidized by molecular oxygen to produce superoxide. Another source of free radicals is the endoplasmic reticulum, in which the reduced form of NADPH cytochrome P450 reductase leaks electrons to molecular oxygen to produce superoxide (8).

Isolated mitochondria in state 4 generate 0.6–1.0 nmol of H_2O_2/min per milligram of protein (9), which is estimated to account for 2% of the oxygen uptake under physiological conditions. The production of superoxide is estimated to be 2–3 nmol of O_2^-/min per milligram of protein. The mitochondria, therefore, are the most important physiological source of O_2^- in animal cells. Similarly, in *Escherichia coli*, the majority of O_2^- is produced by the respiratory chain, with an estimate of 3 O_2^-/10,000 electrons transferred (10). An elegant demonstration of the importance of the electron transport chain in free radical generation was obtained in yeast deficient in manganese-SOD (11). Yeast with a complete absence of electron transport grew normally in hyperoxia, whereas those with an intact electron transport chain showed deficient growth. The main sites at which superoxide is formed are ubiquinone and NADH dehydrogenase. Electrons are transferred one at a time to semiubiquinone, an obligate intermediate for electron

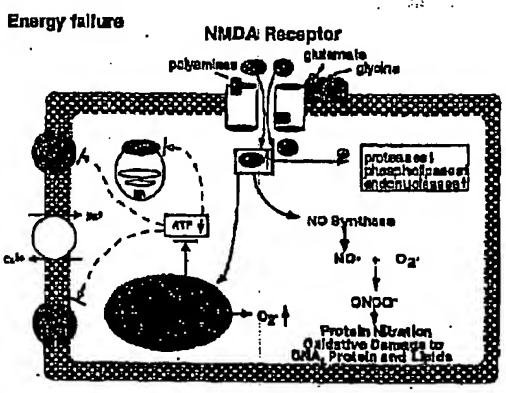

Fig. 2. Mitochondria are a major source of cellular free radicals. Impaired mitochondrial function leads to impaired cellular Ca^{2+} buffering and secondary activation of voltage-dependent NMDA receptors. This can, in turn, lead to further influxes of Ca^{2+}, which are buffered in mitochondria, leading to increased production of O_2^-. Increased Ca^{2+} activates neuronal NO synthase. $NO^{\cdot}$ reacts with O_2^- to generate $ONOO^-$, which can result in both protein nitration, as well as oxidative damage to DNA, proteins, and lipids.

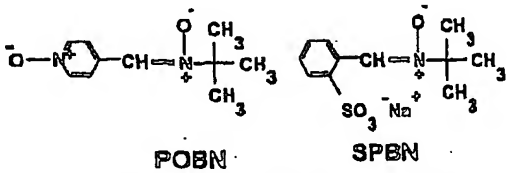

Fig. 3. α-4-pridyl-N-oxide-N-tert-butyl-nitrone and 2-sulfophenyl-N-tert-butyl-nitrone are typical spin trap compounds that react with free radicals to make more stable adducts.

transfer (12). The observations that antimycin (a complex III inhibitor) increases mitochondrial H_2O_2 production with both succinate and NADH-linked substrates but rotenone inhibits production with NADH-linked substrates provide evidence favoring ubiquinone as a site of free radical generation. (13, 14). The effect of antimycin is thought to reflect reduction of cytochrome b, which then leads to increased generation of semiubiquinone. Furthermore, depletion of ubiquinone from mitochondria results in a decrease in H_2O_2 generation. It has been suggested that reconstitution of ubiquinone-depleted mitochondria with ubiquinone restores their ability to generate H_2O_2 (15). However, this remains controversial, and Nohl and Jordan (16) believe that cytochrome b, rather than semiubiquinone, is the source of superoxide (16).

Another source of free radicals is NADH dehydrogenase (17, 18). The production of superoxide from NADH dehydrogenase is thought to involve autooxidation of the NADH dehydrogenase flavoprotein (19) or of the iron-sulfur clusters (17). Production of superoxide at this site is stimulated by rotenone and inhibited by p-hydroxymercuribenzoate (19). Superoxide production by NADH dehydrogenase is about 50% of that produced by ubiquinone (17).

Excitotoxicity and Oxidative Stress

Excitotoxicity refers to neuronal death caused by activation of excitatory amino acid receptors (Fig. 2). Slow or weak excitotoxicity is postulated to result from defects in production of ATP, which may lead to partial neuronal depolarization, followed by activation of voltage-dependent N-methyl-D-aspartate (NMDA) receptors by ambient glutamate concentrations (20). This, in turn, leads to an influx of calcium, which is sequestered into mitochondria. Mitochondrial calcium accumulation contributes to free radical generation. Exposure of mitochondria to Ca^{2+} concentrations similar to those occurring after exposure of neurons to excitotoxins (2.5 μM) leads to mitochondrial generation of hydroxyl and carbon-centered radicals (21). Recent in vitro studies directly linked glutamate-induced increases in cellular calcium to mitochondrial free radical generation. Free radical generation was demonstrated using several redox-sensitive dyes, including dihydrorhodamine, dichlorofluorescein, and dihydroethidine (22–24) . With the latter two dyes, a punctate pattern of fluorescence was seen, consistent with a localization in mitochondria. Furthermore, a recently developed calcium-sensitive dye directly demonstrated that glutamate increases mitochondrial calcium.

The role of $O_2^{\cdot}$ radicals in excitotoxic injury was studied by examining effects on aconitase, a tricarboxylic acid cycle enzyme that contains iron-sulfer clusters inactivated by $O_2^{\cdot}$ (25). Treatment of rat cortical cultures with NMDA, kainic acid (KA), or an intracellular $O_2^{\cdot}$ generator produced a selective and reversible inactivation of aconitase that closely correlated with subsequent cell death. A SOD mimetic blocked both inactivation of aconitase and cell death produced by NMDA and KA.

Markers for Oxidative Damage

The assessment of the role of oxidative damage in neurodegenerative diseases is challenging. Free radicals are extremely short-lived and conventionally detected by reacting them with a nitrone-based spin trapping agent, which produces more stable adducts (Fig. 3). These can then be measured using electron paramagnetic resonance. These techniques, however, are not feasible in humans. Although some studies have used these techniques with blood and postmortem tissue samples, it is not clear that they are reflective of free radical generation in vivo. Novel spin traps, which are incorporated into membranes and show alterations in response to oxidative damage to lipids and protein, have been developed and used in studies of Alzheimer's disease (26). The techniques appear very promising. Another potential means of assessing free radical production in vivo is to use salicylate as a hydroxyl radical trapping compound. Salicylate reacts with hydroxyl radicals to generate two products, 2,3- and 2,5-dihydroxybenzoic acid. These can then be measured using high performance liquid chromatography (HPLC) with electrochemical detection.

In postmortem brain tissue, biochemical markers of oxidative damage to lipids, proteins, or DNA (Table 1) have to be measured. Malondialdehyde is the most commonly used marker of oxidative damage to lipids. Conventional assays using the thiobarbituric acid reaction are hindered by interferences and artifacts. Improved assays using HPLC or mass spectroscopy have now been developed (1). 4-Hydroxynonenal is another marker of lipid peroxidation. Cholesterol and phospholipid hydroperoxides can be measured by sensitive assays using HPLC with chemiluminescence. The most commonly used assay for oxidative damage to proteins is an assay for protein carbonyls. Other markers that may prove useful are dityrosine and oxohistidine. 3-Nitrotyrosine is a marker for peroxynitrite-mediated nitration of proteins (7). It appears to be a useful and specific marker in vivo. Oxidative damage to DNA produces more than 20 base modifications that can be detected by mass spectroscopy. The most frequent is 8-hydroxy-2-deoxyguanosine, which can be found in both tissues and body fluids using HPLC with electrochemical detection (27).

Oxidative Damage in ALS

The observation in 1993 that mutations in CuZnSOD (SOD1) are associated with some familial forms of ALS (FALS) suggested that the disease arises from a perturbation in free radical homeostasis. Although these mutations result in reduced SOD1 activity in vitro, much evidence favors a novel gain of function of the enzyme rather than a loss of function (28). First, no null mutations, which would reduce enzyme activity by preventing protein expression, have been found. Second, the degree of reduction of SOD1 activity does not correlate with either age of onset or duration of disease. Lastly, mice that overexpress high levels of mutant SOD1 develop progressive motor neuron disease, despite normal or elevated SOD1 activity (29–31).

The site of the mutations in the enzyme suggest that they may interfere with normal dimer formation or that they could disrupt zinc binding. This could increase access of the active site copper to either H_2O_2 or $OONO^-$. Recent in vitro studies showed that SOD with two different FALS mutations generates increased hydroxyl radicals (32). Similarly, in vitro studies show that SOD associated with FALS mutations produces increased 3-nitrotyrosine concentrations, as compared with native enzyme (3).

We showed that protein carbonyl groups are increased in the motor cortex of sporadic ALS patients (33). Shaw and colleagues (34) found increased protein carbonyl groups in the spinal cord of sporadic ALS patients. We recently measured concentrations of 3-nitrotyrosine and its major metabolite, 3-nitro-4-hydroxyphenylacetic acid, in the thoracic and lumbar spinal cord of both sporadic ALS patients and three FALS patients with SOD1 mutations. Increased concentrations of both 3-nitrotyrosine and 3-nitro-4-hydroxyphenylacetic acid were found in both the sporadic and the familial ALS patients (35). We also examined whether there was evidence for increased oxidative damage at the cellular level using antibodies to 3-nitrotyrosine, heme oxygenase and malondialdehyde. Both the sporadic and the familial ALS patients show increased staining in anterior horn motor neurons.

A valuable animal model of ALS has been developed by overexpressing FALS SOD1 mutations in transgenic mice. These mice develop progressive motor neuron disease accompanied by microvacuolization of anterior horn cells, swelling and distortion of mitochondria, and swelling of axons (29). We found increased concentrations of 3-nitrotyrosine, as well as increased 3-nitrotyrosine staining, in the motor neurons of these mice (36). Furthermore, the anterior horn cells showed increased staining for both hemoxygenase-1 and malondialdehyde.

These findings, therefore, provide strong evidence that oxidative damage plays a role in the pathogenesis of both sporadic ALS and in ALS associated with SOD1 mutations. The finding of increased 3-nitrotyrosine in both patients with FALS SOD1 mutations, as well as in mice with SOD1 mutations, provides evidence that there is increased reactivity with peroxynitrite in vivo. This could directly contribute to the pathogenesis of the disease by nitrating tyrosines and blocking the effects of neurotrophic factor-induced tyrosine kinases (37), or by nitrating neurofilaments (3). The human neurofilament light chain has 10 tyrosines in the first 96 amino acids that are important for assembly and function of neurofilament protein. Defective neurofilament assembly in transgenic mice leads to aggregates of neurofilaments and impaired axonal transport associated with motor neuron degeneration (38).

Table 1. Biochemical Markers of Oxidative Damage

Lipids	Protein	DNA
Cholesterol hydroperoxides	Carbonyl groups	8-Hydroxy-2-deoxyguanosine
Phospholipid hydroperoxides	Oxohistidine	
4-Hydroxynonenal	Dityrosine	
Malondialdehyde	3-Nitrotyrosine	

Oxidative Damage in Alzheimer's Disease

There are several potential sources of oxidative damage in Alzheimer's disease. β-Amyloid itself may be able to generate free radicals, and its toxicity to cultured neurons and to endothelial cells is blocked by antioxidants (39, 40). There is also evidence that β-amyloid can stimulate inflammatory cells (microglia) to produce free radicals, including NO (41). The amyloid precursor protein can reduce Cu^{2+} to Cu^{+}, which can then contribute to oxidative damage by reacting with hydrogen peroxide to generate hydroxyl radicals (42). Down's syndrome patients develop premature Alzheimer's disease, and cultured Down's syndrome neurons show decreased survival and increased free radical production (43). Lastly, substantial evidence exists for a defect in cytochrome oxidase in Alzheimer's disease. A cytochrome oxidase defect can be transferred from Alzheimer's disease platelets to mitochondria-deficient cell lines, which then show increased free radical production (44).

Substantial evidence for increased oxidative damage in Alzheimer's disease exists from studies of postmortem brain tissue. There are consistent increases in lipid peroxidation as assessed by increases in malondialdehyde concentrations. An increase in protein carbonyl groups was shown relative to young controls. Studies using novel spin trapping techniques showed oxidative damage to both lipids and protein (26). We showed a 50% increase in 8-hydroxy-2-deoxyguanosine concentrations in nuclear DNA and a threefold increase in mitochondrial DNA in Alzheimer's disease cerebral cortex (27).

Recent studies looked for evidence of oxidative damage at the cellular level. Both neurofibrillary tangles and senile plaques stain with antibodies to SOD or catalase (45, 46). Antibodies to advanced glycation end products stain neurofibrillary tangle bearing neurons

(47, 48). These neurons also show staining with antibodies to malondialdehyde and hemoxygenase-1 (47–49). Hemoxygenase-1 is induced by oxidative stress in cultured neurons and by traumatic brain injury in vivo. Hemoxygenase-1 mRNA levels are increased in Alzheimer's disease postmortem brain tissue (50). Two distinct antibodies that recognize carbonylated neurofilaments label Alzheimer's disease neurofibrillary tangles, neuropil threads and granulovacular degeneration (51). Furthermore, antibodies to protein carbonyls label neurofibrillary tangle bearing neurons (52). We found increased staining for hemoxygenase-1, malondialdehyde, and 3-nitrotyrosine in neurofibrillary tangle bearing neurons.

Oxidative Damage in Parkinson's Disease

Much interest has focused on the possibility that oxidative damage may play a role in the pathogenesis of Parkinson's disease. Mitochondrial complex I defects have been found in Parkinson's disease substantia nigra, muscle, and platelets. A recent study showed a decreased PCr/Pi ratio in Parkinson's disease muscle consistent with an energetic defect can now be made (53).

Direct evidence for oxidative damage comes from two studies showing increased malondialdehyde in substantia nigra in Parkinson's disease. This was confirmed by a study showing increased cholesterol hydroperoxides in the substantia nigra in Parkinson's disease. Furthermore, concentrations of 8-hydroxy-2-deoxyguanosine were increased threefold to fourfold in Parkinson's disease caudate and substantia nigra (54). A more indirect measure of oxidative stress is reduced glutathione concentration. Decreased glutathione concentrations in Parkinson's disease substantia nigra have been found in four separate studies. Furthermore, reduced glutathione concentrations are found in patients with incidental Lewy bodies in the substantia nigra, which may be a presymptomatic phase of Parkinson's disease (55). An immunohistochemical study showed increased 4-hydroxynonenal protein adducts, a marker of lipid peroxidation, in Parkinson's disease substantia nigra neurons (56).

Further evidence implicating oxidative damage in the pathogenesis of Parkinson's disease comes from studies of MPTP neurotoxicity. In both humans and experimental animals, MPTP produces a Parkinsonian syndrome that closely mimics findings that occur in idiopathic Parkinson's disease. The pathogenesis of the lesions involves inhibition of mitochondrial complex I by 1-methyl-4-phenylpyridinium (MPP^+), the active metabolite of MPTP. MPP^+ results in increased free radical generation in vivo, and the toxicity of MPTP is attenuated in mice overexpressing SOD (57). We showed that 7-nitroindazole, a relatively selective inhibitor of the neuronal isoenzyme of NOS, produces dose-dependent inhibition of MPTP neurotoxicity accompanied by reduced striatal concentrations of 3-nitrotyrosine (58). These results were recently confirmed and extended by showing that mice deficient in neuronal NOS are partially resistant to MPTP toxicity (59). Furthermore, 7-nitroindazole produced complete protection against MPTP neurotoxicity in baboons (60). These findings show that peroxynitrite plays a role in MPTP neurotoxicity and, by implication, in Parkinson's disease.

Oxidative Damage in Huntington's Disease

Substantial evidence for a defect in energy metabolism exists in Huntington's disease. A recent study showed an interaction between huntingtin, the protein encoded by the gene defect, and glyceraldehyde-3-phosphate dehydrogenase (61). There appears to be an inhibition of the enzyme with increasing numbers of glutamine repeats in the huntingtin protein. An increase in CAG repeats encoding glutamine is known to be responsible for the disease. Impaired glyceraldehyde-3-phosphate dehydrogenase activity could lead to increased oxidative damage as a consequence of secondary excitotoxicity.

There is a paucity of studies examining markers of oxidative damage in Huntington's disease. We found increased nuclear 8-hydroxy-2-deoxyguanosine concentrations in the caudate nucleus and in mitochondrial DNA of frontal cortex (62). The most compelling evidence implicating oxidative stress in the pathogenesis of Huntington's disease comes from animal studies. We showed that systemic administration in both rodents and primates of the succinate dehydrogenase inhibitor 3-nitropropionic acid produces selective striatal degeneration closely resembling Huntington's disease (63). These lesions are accompanied by increased oxidative damage as assessed by 2,3- and 2,5-DHBA/salicylate ratios, concentrations of 8-hydroxy-2-deoxyguanosine, and concentrations of 3-nitrotyrosine (58). The lesions are attenuated in mice overexpressing SOD, as well as by free radical spin traps and by the neuronal NOS inhibitor 7-nitroindazole (58, 64). Protection is accompanied by attenuation of the increases in markers of oxidative damage.

Conclusions

The evidence for a role of oxidative damage in neurodegenerative diseases is becoming increasingly compelling. Improved biochemical methods are being developed for examining oxidative damage in brain tissue, and new methods are being developed for assessing oxidative damage at a cellular level. A strong case for a role of oxidative damage can now be made in both sporadic and familial ALS with SOD1 mutations. Evidence for oxidative injury in the pathogenesis of other neurodegenerative diseases is increasing rapidly.

The critical issue in the assessment of oxidative injury in neurodegenerative diseases is whether it plays a causative role or is merely a passive bystander. In animal studies, this can be ascertained by therapeutic interventions. We found that free radical spin traps are effective in attenuating neuronal injury produced by both MPTP, a model of Parkinson's disease, and by 3-nitropropionic

acid or malonate, which model Huntington's disease. Furthermore, neuronal NOS inhibitors are very effective in both these models. Coenzyme Q10, a free radical scavenger as well as an enhancer of mitochondrial function, has neuroprotective effects against MPTP, malonate, and the transgenic mouse model of ALS (58, 64). Vitamin E produced clinical improvement in transgenic ALS mice but did not alter survival (65).

Whether these observations will translate into useful therapies for human neurodegenerative diseases is unknown. The development of new, improved antioxidants may hold great promise as a therapy that could slow or halt the progression of neurodegenerative diseases.

Acknowledgment

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Age-related memory decline and longevity under treatment with selegiline.

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The MAO-B inhibitor selegiline is used in the treatment of Parkinson's disease. Further, beneficial effects in Alzheimer's disease have also been described as well as neuroprotective effects, increased longevity and an attenuation of age-related cognitive decline in experiments using rats. Our studies in mice and Syrian hamsters aim at the question whether the effects of selegiline reported in the rat can be generalized to other species. Aged female NMRI-mice (23 mo.) treated with selegiline (0.25 mg/kg, i.p., 3 times a week for 2-3 weeks) showed no treatment effect in the Morris water maze and in passive avoidance learning after 2 and 3 weeks of treatment. However, Syrian hamsters chronically treated with selegiline (0.05 mg/kg/day in the food, starting at 12 months old) showed a 3 month delay in the age-related decline of spontaneous alteration behavior, a measure of longer-term memory, compared to untreated controls. Since treated hamsters also show increased longevity (study still in progress) the data suggest a protective effect of a chronic treatment with selegiline against age-related cognitive and physical decline.

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